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(71) Applicant: **BIO DEFENCE INSTITUTE CO., LTD.**  
**Syouei-kannai Building, 22,**  
**Sumiyoshi-cho 2-chome,**  
**Naka-ku**  
**Yokohama-shi, Kanagawa(JP)**

(72) Inventor: **Egawa, Kohji**  
**1-11, Kamiyouga 3-chome,**  
**Setagaya-ku**  
**Tokyo(JP)**  
Inventor: **Sato, Ichiei**  
**2413, Ishihara-cho**  
**Takasaki-shi, Gunma(JP)**

(74) Representative: **Hering, Hartmut, Dipl.-Ing.**  
**Patentanwälte**  
**Berendt, Leyh & Hering**  
**Innere-Wiener-Strasse 20**  
**D-81667 München (DE)**

(54) **Anti-tumor method and anti-tumor agent.**

(57) An anti-tumor method and an anti-tumor agent using immunoreaction specific to tumor antigens expressed on the surface of tumor cells. A human non-classical histocompatibility class 1 antigen, an antibody which identify this antigen, or a cytotoxic lymphocyte is administered to a cancer patient as an immunotherapy to induce resistance against tumors.

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prepared in the above step f, or lymphocytes obtained from peripheral blood of tumor-bearing individuals, in vitro under existence of the lymphocytes obtained in the above step f;

2. establishing the objective cytotoxic clones obtained from these lymphocytes using a limiting dilution method; and

5 3. confirming specificity of identification by measuring cytotoxicity against the lymphocytes prepared in the above step f and control lymphocytes which are not introduced with cDNA.

## SUMMARY OF THE INVENTION

10 The present invention provides an anti-tumor method wherein non-classical histocompatibility class 1 antigen, an antibody which identifies said antigen or a cytotoxic splenocyte is administered, and an anti-tumor agent comprising said antigen as an effective component.

The present invention is described in detail referring to examples in the followings, although the invention is not limited to those examples.

## 15 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph showing existence of Qa-2 antigen on tumor cell strains derived from H-2<sup>k</sup> mice;

20 Fig. 2 is a graph showing results of testing complement-dependent cytotoxic activity of the anti-Qa-2 monoclonal antibody (mAb) and fast-protein-liquid-chromatography (FPLC)-purified material;

Fig. 3 illustrates results of autoradiography of the <sup>125</sup>I-labelled FPLC-purified material;

Fig. 4 is a graph showing adsorption of regressor serum (RS) activity with an anti-IgD-Sepharose column;

Fig. 5 is a graph showing existence of IgD specific to Qa-2 antigen in RS by enzyme-linked immunosorbent assay (ELISA);

25 Fig. 6 illustrates specificities of oligonucleotides used as primers in polymerase chain reaction (PCR);

Fig. 7 shows a result of DNA-DNA hybridization analysis;

Fig. 8 shows a result of DNA-RNA hybridization analysis;

Fig. 9 shows amplification of BW5147 cDNA by PCR;

Fig. 10 shows reactivity to Qa-2-specific mAb of a fusion protein derived by *E. coli*; and

30 Fig. 11 shows nucleotide amplification of cDNAs prepared from human tumor cell strains.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

### Example 1:

### 35 [Materials and Methods]

#### (1) Animals

40 Thirteen syngeneic mouse strains with known Qa, TL and Ly phenotypes (listed in Table 1) were used (KLEIN, J., FIGUEROA, F., DAVID, C.S.; H-2 Haplotypes, gene and antigens: Second listing, Immunogenetics, 1983, 17:553, MCKENZIE, IFC., POTTER, T.; Murine lymphocyte surface antigens, Adv Immunol, 1979, 27:179).

45 B6, B6.K1 and B6.K2 are congenic with respect to the Qa-1, Qa-2 and Qa-3 antigens (STANTON, TH., BOYSE, EA.; A new serologically defined locus, Qa-1, in the Tla-region of the mouse, Immunogenetics, 1976, 3:525). C3H/He and C3H-Ly6.2 are congenic regarding to allotypes of the Ly6 antigen. The B6.K1, B6.K2 and C3H-Ly6.2 strains were provided by Dr. T. Takahashi of the Aichi Cancer Center Research Institute and maintained by the present inventors.

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## (5) Adsorption of RS

For use of RS in adsorption tests,  $\gamma$ -globulins were depleted from RS by precipitation with ammonium sulfate at 0.35 saturation and then by starch gel zone electrophoresis as described below. The resulting material was used as  $\gamma$ -globulin-depleted RS although it may still contain some  $\gamma$ -globulins.

$\gamma$ -globulin-depleted RS was diluted to 100 times the original volume of RS with RPMI-1640 medium supplemented with 7 % fetal calf serum (FCS). One ml of this solution was mixed with  $2 \times 10^7$  lymphocytes (splenocytes or mixture of splenocytes and mesenteric lymphonode cells), which had been freed of erythrocytes by the ammonium chloride method (BOYLE, W.; An extension of cytotoxins, Transplantation 1968, 6:761) and washed three times with Eagle's minimum essential medium (MEM), or with  $(4 \text{ to } 6) \times 10^5$  ascites tumor cells washed in the same way.

The mixture was incubated at  $25^\circ\text{C}$  for 30 min and then at  $0^\circ\text{C}$  for 60 min. The cells were removed by centrifugation at 1,000 rpm for 5 min. In case of the adsorption with lymphocytes, the procedure was done twice. The resulting supernatant was used as the adsorbed RS.

## (6) Assay of cytotoxicity

Splenocytes of C3H/He mice, obtained 3 days after intraperitoneal transplantation of MM2 cells, were used as the effector cells of the RS-dependent in vitro cytotoxicity reaction (TANINO T, EGAWA K; Regressor serum factor-dependent nonspecific killers in tumor-bearing mice). The effector cells ( $1 \times 10^5$ ) were mixed with  $1 \times 10^4$  of  $^{51}\text{Cr}$ -labelled target cells in 0.5 ml RPMI-1640 medium supplemented with 7 % FCS.

In adsorption tests, the  $\gamma$ -globulin-depleted RS before or after the adsorption was added at a final concentration of 500  $\times$  dilution of the original RS. MM2 cells, various lymphoblasts and L cell transformants were used as the target cells. The mixture was incubated for 18 h in case of tumor cells, 5 to 7 h in case of lymphoblasts and 10 h in case of L cell transformants at  $37^\circ\text{C}$  in a  $\text{CO}_2$  incubator.

After the incubation, the radioactivity released from the cells into the supernatant and the radioactivity remaining in the cells were determined and the cytotoxicity was expressed in terms of percentage specific lysis. All determinations were carried out in duplicate. In the adsorption tests, results of repeated or different experiments were normalized by expressing them in terms of percentage decrease of the RS activity.

Lymphoblasts were prepared by culturing splenocytes from various mice at a cell density of  $1 \times 10^6$  cells/ml for 2 days in RPMI-1640 medium supplemented with 10 % FCS and then for 3 days in the medium containing 5  $\mu\text{g/ml}$  Concanavallin A (Sigma, St. Louis).

$\text{L}^{\text{Q7b/Kb}}$  and  $\text{L}^{\text{Kb}}$  cells were established by transfection of  $\text{Q7b/Kb}$  hybrid gene DNA and H-2K<sup>b</sup> gene DNA into  $\text{L}^{\text{tk-}}$  cells.  $\text{L}^{\text{Q7b/Kb}}$  cells express the  $\alpha 1$  and  $\alpha 2$  domains of the  $\text{Q7b}$  gene product and  $\alpha 3$  domain of H-2K<sup>b</sup> molecule. The  $\alpha 1$  and  $\alpha 2$  domains are mainly responsible for immunologically defined Qa-2 specificity of H-2<sup>b</sup> mouse lymphocytes (WANECK, G.L., SHERMAN, D.H., CLAVIN, S., ALLEN, H., and FLAVELL, R.A.; Tissue-specific expression of cell-surface Qa-2 antigen from a transfected Qb<sup>7</sup> gene of C57BL/10 mice, J. Exp. Med., 1987, 165:1358).  $\text{L}^{\text{Kb}}$  cells express the H-2<sup>b</sup> molecule on their surfaces.

Complement-dependent cytotoxic activity was assayed by the dye exclusion method with mesenteric lymphonode cells as the target cells. The cells ( $5 \times 10^5$ ) were suspended in 100  $\mu\text{l}$  of MEM containing 5 % FCS and the antibody. The suspension was incubated at room temperature for 90 min. The cells were washed twice with MEM, incubated in 100  $\mu\text{l}$  of MEM containing complement at  $37^\circ\text{C}$  for 45 min, washed twice with MEM again, added with saline containing 0.2 % trypan blue and examined under the microscope. The percentage lysis was calculated and the value in the absence of antibody was subtracted as the background.

## (7) Serum protein fractionation

A serum protein subfraction precipitated between 0.35 and 0.50 saturation of ammonium sulfate at  $4^\circ\text{C}$  was fractionated by starch-gel zone electrophoresis using a starch block of  $10 \times 40 \times 1.5$  cm buffered with 0.07 M veronal buffer (pH 8.6).

The hydrolyzed starch used for gel electrophoresis was purchased from Connaught Laboratories, Willowdale, Ontario.

After electrophoresis for 24 h at  $4^\circ\text{C}$  at 35 mA, the gel was cut into 1-cm-wide blocks and each gel portion was eluted with 10 ml water. The resulting  $\beta$ -globulin fraction was dialysed against 0.01 M TRIS-HCl buffer (pH 8.6), applied on a 1 ml Mono-Q column of fast protein liquid chromatography (FPLC) apparatus.

## [Results]

## (1) Recognition specificity of RS factors

It has been reported that splenocytes, obtained from C3H/He mice bearing ascites tumor cells 3 days after inoculation, lyse various syngeneic (MM2, MM46 etc.) and allogeneic (Meth A, EL-4 etc.) tumor cells by an RS-dependent cellular cytotoxic reaction. Some syngeneic tumor cells (MM48, X5563 etc.) were lysed less strongly by the reaction (TANINO, T., EGAWA, K.; Regressor serum factor-dependent non-specific killers in tumor-bearing mice).

Such activity of RS was adsorbed to various extents by those tumor cells which were susceptible to the reaction. However, the activity of RS was not adsorbed by those tumor cells which were resistant to the reaction. Susceptibility of the allogeneic tumor cells in general leads to a possibility that the active components in RS (referred as RS factors) recognize allogeneic antigens which might be expressed illegitimately on the tumor cell surface. To test this possibility, RS was adsorbed by lymphocytes obtained from mice of various strains. Results are shown in Table 2.

Table 2

Adsorption of RS activity with lymphocytes from mice of various strains				
Origin of lymphocytes used for adsorption	H-2 haplotype	Target cells		% Decrease of RS activity*
		MM2	Meth A	
C3H/He	k	3.4 ± 2.0	6.5 ± 4.3	
CBA	k	2.1 ± 1.8	5.5 ± 4.1	
CE	k	23.5 ± 1.4	3.6 ± 0.0	
AKR	k	40.5 ± 2.7	6.5 ± 6.5	
C58	k	47.0 ± 2.7	24.2 ± 8.2	
C57BL/6	b	68.7 ± 16.1	74.3 ± 12.8	
BALB/c	d	41.8 ± 9.7	43.3 ± 3.1	
DBA/2	d	67.9 ± 11.3	79.6 ± 8.7	
DBA/1	q	46.8 ± 14.8	68.9 ± 12.2	
A/J	a	49.8 ± 6.5	84.5 ± 9.9	

\* Means ± SE of the results of 2 experiments. Percent specific lysis of MM2 and Meth A cells in the presence of unadsorbed material was  $33.2 \pm 4.4$  and  $28.4 \pm 4.0$ , respectively.

As shown in Table 2, the activity of RS, assayed using MM2 target cells, was adsorbed to various extents by lymphocytes with H-2 haplotypes of k (CE, AKR and C58) and those with H-2 haplotypes other than k (B6, BALB/c, DBA/2, DBA/1 and A/J), but not by lymphocytes from C3H/He and CBA mice (both H-2<sup>k</sup>). Similar but not identical pattern of adsorption was observed when Meth A cells were used as the target cells of the cytotoxicity reaction. The results showed that adsorption of the RS factors occurred when allotypes of one or some of the antigens in the Ly group of lymphocytes were different from those of C3H/He, or when one or some of Qa/TL antigens were expressed on the splenocytes. Results which further support this were obtained by double-absorption experiments and is shown in Table 3.

Table 4

RS-dependent cell-mediated lysis of various lymphoblasts		
Origin of the lymphoblasts	RS target	
	-	+
	% Specific lysis*	
C3H/He	0.3 ± 1.2	4.2 ± 5.2
C3H-Ly6.2	0.0 ± 0.6	30.5 ± 12.5
CE	0.4 ± 0.3	20.1 ± 3.5
C58	0.2 ± 0.4	31.6 ± 5.1
C57BL/6	5.2 ± 4.8	37.5 ± 12.0
B6.K1	0.1 ± 0.2	33.2 ± 4.9
B6.K2	0.1 ± 0.3	37.1 ± 7.0
DBA/1	4.8 ± 5.3	32.8 ± 2.7
DBA/2	2.3 ± 3.2	31.4 ± 1.5
A/J	0.3 ± 0.2	36.7 ± 1.2

\* Means ± SE of the results of two experiments. Ig-depleted RS was added at a final concentration of 500 × dilution.

seems to account partly for the wide target selectivity of the RS-dependent reaction.

### (3) Characteristics of the Qa-2-specific RS factor

Among multiple factors recognizing allo-antigens presumed to be present in RS, the specificity of a factor to Qa-2 was most clearly demonstrated as described above. The inventors therefore focused their attention on this factor and tried to characterize it further.

By ammonium sulfate precipitation and preparative electrophoresis of the serum protein, the activity to support the cell-mediated lysis of B6 lymphoblasts, but not of B6.K1 lymphoblasts, was found mainly in the  $\beta$ -globulin fraction. This fraction was then fractionated by FPLC using the Mono-Q column.

The activity was associated with the material eluted at 0.30 M NaCl, whereas almost all of the mouse mAbs of the IgG subclasses and those of the IgM class were eluted at around 0.25 M and 0.35M NaCl positions, respectively.

The semi-purified material was also tested for complement-dependent cytotoxic activity against Qa-2<sup>+</sup> lymphocytes (Fig. 2). Complement-dependent lysis of B6 mesenteric lymphnode cells by the FPLC-purified material was not detected even when 35  $\mu$ g protein was used per assay. On the other hand, anti-Qa-2 mAb of the IgG2b class in the presence of complement lysed the same target cells at an antibody concentration as low as less than 1  $\mu$ g protein per assay.

To further characterize the Qa-2-specific RS component, the FPLC-purified material was radiolabelled with <sup>125</sup>I, preadsorbed with C58 lymphocytes, adsorbed to the surface of Qa-2<sup>+</sup> cells and analysed by SDS-PAGE or by two dimensional PAGE followed by autoradiography (Fig. 3).

In Fig. 3, A shows one-dimensional SDS-PAGE under non-reducing (a) and reducing (b) conditions, using a 7.5 % polyacrylamide gel; and B shows pattern of two-dimensional PAGE.

Specific binding of about 2 % of the total radioactivity to the cells was observed. The relative mass of the bound material under non-reducing condition was estimated to be 160 kDa. Under reducing conditions, it showed bands with relative masses of approximately 50 kDa and 25 kDa. These results revealed that the material had a heavy and light chain structure similar to that of IgG. The two dimensional gel electrophoresis of the labelled material showed that it was composed of four or more components whose isoelectric points (PI) ranged approximately from 5.5 to 6.5 and all of which had heavy and light chain structures.

It is known that IgD are found in the  $\beta$ -globulin fraction of serum proteins and comprise a group of acidic glycoproteins (ISHIHARA, E., TEJIMA, Y. TAKAHASHI, R. TAKAYASU, T., SHINODA, T.; Structure and location of a sparagine-linked oligosaccharides in the Fc region of a human immunoglobulin D, Biochem Biophys Res Comm, 1983, 110:181), whereas IgGs in general are basic proteins. IgD has a structure composed of heavy and light chains like IgGs, but does not interact with C1q of the complement system (SPIEGELBERG, HL.; Immunoglobulin D (IgD), Methods in Enzymology, 1985, 116:95, SPIEGELBERG, HL.; The structure and biology of human IgD, Immunological Rev, 1977, 37:3).

It has also been reported that there are two forms of mouse serum IgD. One has a molecular mass of about 170-200 kDa, while the other, which lacks the C1 domain of the heavy chains, has a molecular mass of approximately 150-160 kDa (FINKELMAN FD., KESSLER, SW., MUSHINSKI, JF., POTTER, M.; IgD-secreting murine plasmacytomas: Identification and partial characterization of two IgD myeloma proteins, J Immunol, 1981, 126:680, MOUNTS, JD., MUSHINSKI, JF., OWENS, JD, FINKELMAN, FD.; The in vivo generation of murine IgD-secreting cells is accompanied by deletion of C  $\mu$  gene and occasional deletion of the gene for the C $\delta$ 1 domain, J Immunol, 1990, 145:1583). Variety in the sugar moieties of a human monoclonal IgD (NIG-65 myeloma protein) seems to cause heterogeneity in the isoelectric point between 5.6 and 6.8.

These characteristics agree with those of the serum component with Qa-2 specificity. For this reason, the inventors speculated that the Qa-2 specific component is an IgD.

To test this possibility, the FPLC-purified material after adsorption with L<sup>Kb</sup> cells was further adsorbed with Sepharose 4B conjugated with each one of the antibodies specific to various mouse immunoglobulin subclasses. In Fig. 4-A, are shown the cell-mediated lytic activity to Q7b/Kb cells of the material before the adsorption ( $\Delta$ ), after adsorption with L<sup>Kb</sup> ( $\blacksquare$ ) and after further adsorption with the Sepharose 4B ( $\bullet$ ). The activity demonstrated by using L<sup>Q7b/Kb</sup> as the target cells was adsorbed considerably with Sepharose 4B conjugated with anti-IgD.

The material retained on and eluted from the anti-IgD column had significant activity to support lysis of L<sup>Q7b/Kb</sup> cells (Fig. 4-B). The arrow in Fig. 4-B shows the position of the start of the elution.

The activity was not significantly adsorbed with Sepharose 4B conjugated with either one of anti-IgG1,

Cancer cells ( $2 \times 10^6$ ) were incubated at  $37^\circ\text{C}$  for 60 min in 30  $\mu\text{l}$  of D-MEM (pH 7.5) containing 0.1 mU of PI-PLC and 1 mg/ml of ovalbumin. After the incubation, the cells were washed twice with D-MEM and used for detection of cell surface antigens.

#### 5 (5) Immunofluorescence analysis

Detection of cell surface Qa-2 and Thy1 antigens was carried out by flow cytometry. Briefly, the cells were reacted with Qa-2-specific mAb and then with the FITC-labelled  $\text{F(ab')}_2$  fraction of goat antibody specific to mouse immunoglobulins.

10 In the cases of lymphocytes, T cells were enriched by passing through a Nylon wool column and cell surface immunoglobulins of contaminating B cells were blocked by treating with Fab' fragment of the antibody. The FITC-labelled cells were fixed with 1 % paraformaldehyde and analyzed using the fluorescence activated cell sorter (FACS III, Becton Dickinson, CA).

#### 15 (6) Preparation of cDNA

Total cellular RNA was extracted from tumor cells and thymocytes by the guanidine isothiocyanate method (SCHIBLER, U., TOSI, M., PINETT, A.-C., FABIANI, L., and WELLAUER, P.K.; Tissue-specific expression of mouse alpha-amirase genes, *J Mol Biol*, 1980, 142:93), and was enriched for mRNA by isolating poly(A)<sup>+</sup> RNA on oligo(dT)cellulose (Boehringer Mannheim GmbH, Germany) (AVIV, H., and LEDER, P.; Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid cellulose, *Proc Natl Acad Sci USA*, 1972, 69: 1408). Synthesis of cDNA from the mRNA was carried out using a cDNA synthesis kit (cDNA plus, Amersham Corp., Arlington Heights, IL) with oligo-dT as a primer.

#### 25 (7) Oligonucleotide primers and probes

Based on the reported nucleotide sequences of genes in the Qa-2, 3 region of C3H/He (WATTS, S., DAVIS, A.C., GANT, B. WHEELER, C., HILL, L. and GOODENOW, R.S.; Organization and structure of the Qa genes of the major histocompatibility complex of the C3H mouse, Implications for Qa function and class I evolution, *EMBO J*, 1989, 8:1749), eleven 20mer oligonucleotides were prepared on a DNA synthesizer (Applied Biosystems model 391) and used as primers for polymerase chain reaction (PCR). Their sequences and specificities are shown in Table 6 and Fig. 6.

Oligonucleotides Nos. 1, 2, 3, 4, 5 and 6 were complementary to specific antisense sequences found in the first exons of each of the H-2D<sup>K</sup>, Q1<sup>K</sup>, Q2<sup>K</sup>, Q4<sup>K</sup>, Q5<sup>K</sup> and Q10<sup>K</sup> genes, respectively. Nos.10 and 11 were complementary to a Q5<sup>K</sup>-specific sense sequence in exon 8 and exon 7, respectively. Nos. 7, 8 and 9 were complementary to sequences common to all class I genes. Oligonucleotides Nos. 12 to 17, complementary to Nos. 1 to 6 respectively, were also synthesized and used as probes in DNA-RNA hybridization experiments.

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## (11) DNA-RNA hybridization

Poly(A)<sup>+</sup> RNA was prepared as described above from AKR thymocytes and BW5147 cells, denatured with glyoxal, electrophoresed through 1.2 % agarose gel containing 20 mM phosphate buffer (pH 7.4) and transferred onto a Nylon filter. The filter was dried at 37 °C and incubated for 1 h at 60 °C in 2 ml of the hybridization buffer containing 100 µg salmon sperm DNA.

Oligonucleotide probes, 5'-terminus of which had been labelled with <sup>32</sup>P, was then added to the incubation. After incubating at 42 °C overnight, the filter was washed twice with 2 × SSC for 15 min at room temperature and twice with 2 × SSC containing 0.1 % SDS for 30 min at 50 °C. The filter was then rinsed with 0.2 × SSC and exposed to X-ray film using an intensifier screen.

(12) Synthesis of Q5<sup>K</sup> protein and Western blotting

DNA corresponding to exons 1 to 4 of the Q5<sup>K</sup> gene which had been amplified from BW5147 cDNA was cloned into pUC119 and sequenced as described above. A colony was selected which had the insertion in the right direction and in frame downstream of the lacZ promoter. This colony was grown in L both containing Ampicillin until the optical density at 600 nm reached 0.3. IPTG was added to the culture to 50 µM and culturing continued until the optical density became 0.75.

The cells were harvested, precipitated with 10 % trifluoroacetic acid, washed with acetone and dissolved by heating for 30 min at 100 °C in 25 mM Tris-hydrochloride (pH 6.8) containing 2 % SDS, 5 % glycerol, 0.0125 % BPB and 2.5 % 2-mercaptoethanol.

The dissolved material was electrophoresed through a 12 % polyacrylamide gel containing 0.1 % SDS and transferred onto polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, NA) by electroblotting in a solution composed of 25 mM Tris base, 192 mM glycine, 20 % methanol and 0.02 % SDS. The membrane was then rinsed with 0.15 M sodium chloride containing 0.2 % Tween 20 and buffered with 10 mM Tris-hydrochloride (pH 7.6) (TTBS), incubated for 2 h at 37 °C in TTBS containing 10 % goat serum and 10 % horse serum.

The membrane was rinsed with water and incubated overnight at room temperature in 200 × diluted mouse ascites containing mAb 59 in 5 ml of the same medium. After the incubation, the filter was rinsed 3 times with TTBS and reacted for 1 h at 37 °C with peroxidase-labelled anti-mouse IgG (Amersham) in saline buffered with 10 mM Tris-borate (pH 7.6). The membrane was again washed 3 times with TTBS and treated for coloration in 50 ml of TTBS containing 40 mg of 3,3'-diaminobenzidine-tetrahydrochloride (Wako Chemicals, Osaka, Japan) and 15 µl of 30 % hydrogen peroxide.

## [Results]

(1) Characteristic of the Qa-2 antigen expressed on tumor cells derived from H-2<sup>K</sup> mice.

In the report of TANINO, T., SEO, N., OKAZAKI, T., NAKANISHI-ITO, C., SEKIMATA, M., and EGAWA, K; Humoral responses to Qa-2 and other tumor antigens in mice, (Submitted for publication in Immunogenetics), it was demonstrated that most tumor cell lines (7 out of 9 cell lines tested) derived from H-2<sup>K</sup> (Qa-2<sup>+</sup>) mice express the Qa-2 antigen detected by the membrane immunofluorescence method using anti-Qa-2 antiserum or Qa-2-specific mAb 141-15.8.

It was found, however, that the Qa-2 antigen was not detected on these cells in general when another Qa-2-specific mAb 34-1.2 was used. Qa-2-specificity of the newly obtained mAb 59 was examined and compared to those of mAbs 141-15.8 and 34-1.2. The result is shown in Table 7.



Table 8

## Susceptibility of Qa-2 and Thyl antigens to PI-PLC treatment

A.				
Antibody				
-----				
	mAb 141-15.8		non-related mAb	
-----				
PI-PLC treatment				
-----				
Cell	-	+	-	+
-----				
Mean fluorescence intensity				
B6 lymphocyte	152.0	59.7	30.6	29.4
BW5147	85.1	96.4	35.8	36.2
MM2	88.2	98.0	73.2	74.6
FM3A	108.0	109.3	71.1	72.3
MH134	101.2	102.9	67.9	72.5
-----				
B.				
Antibody				
-----				
	anti-Thyl1.1		anti-Thyl1.2	
			non-related mAb	
-----				
PI-PLC treatment				
-----				
Cell	-	+	-	+
-----				
Mean fluorescence intensity				
AKR thymocyte	193.0	98.0		34.5
B6 thymocyte		176.0	79.7	40.1
BW5147	176.0	95.1		

From these observations, it is evident that the Qa-2 antigen expressed on tumor cells derived from H-2<sup>k</sup> mice (Qa-2<sup>k</sup> antigen) is distinct immunologically and biochemically from that expressed on H-2<sup>b</sup> normal lymphocytes. The Qa-2<sup>k</sup> antigen can be defined as a PI-PLC-resistant molecule expressed on tumor cells derived from H-2<sup>k</sup> mice and which is reactive to mAbs 59 and 141-15.8 but not to mAb 34-1.2.

culturing the cells under the existence of IPTG.

As shown in Fig. 10, the obtained *E. coli* showed the induced synthesis of a peptide with molecular mass of about 35 kDa, which was the value expected from the fusion protein. This 35 kDa protein reacted with peroxidase-labelled anti-Qa-2 mAb 59. The protein was not detected under identical conditions when peroxidase-labelled anti-Qa-2 mAb 34-1.2 was used.

From these results, the inventors concluded that the Qa-2<sup>k</sup> antigen on the surface of BW5147 cells, which was detected by mAb 59 but not by mAb 34-1.2, and which was resistant to treatment with PI-PLC, was the product of the Q5<sup>k</sup> gene.

### 10 Example 3:

From the experiments of the above Examples, it is evident that activation of Q5<sup>k</sup> gene in the Qa/T1a region is admitted, the Q5 gene product has common antigenicity (cross-reactivity) with normal Qa-2 lymphocyte antigen, and further, response of the Qa-2<sup>-</sup> mice bearing tumor cells against the Q5 gene product expressed by the tumor cells can target also the Qa-2 antigen (the product of Q7 gene) on the surface of the allogeneic normal lymphocytes.

Therefore, the present inventors made experiments for inducing resistance against tumor cells by immunity using the Qa-2 antigen.

### 20 [method]

Qa-2<sup>-</sup> mice were immunized by normal lymphocytes of Qa-2<sup>+</sup> allogeneic mice. As Qa-2<sup>-</sup> mice, (C3H/He × B6.K1) F<sub>1</sub> mice were used, and administered intraperitoneally with splenocytes of C57BL/6 mice to immunize specifically to Qa-2 antigen. As tumor cells, Qa-2<sup>+</sup> tumor cells from C3H/He mice were used.

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### [Results]

(1) By the above immune treatment specific to Qa-2 antigen, resistance against tumor cells, such as MM2 of C3H/He mice origin, was induced in the F<sub>1</sub> mice. Namely, while transplantation of  $2 \times 10^5$  tumor cells was effectuated in the control nontreated group, transplantation was not effectuated in the immunized mice.

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Further, when the F<sub>1</sub> mice were immunized by lymphocytes of B6.K2 mice instead of C57BL/6 mice, same results were obtained as shown in the following Table 9.

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Table 9

mouse	rate of transplantation of MM2 cells
mouse immunized against Qa-2 antigen	0/10*
Control untreated mouse	10/10

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\* denominator: total number of mouse used

numerator : number of mouse to which transplantation was effectuated

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(2) Splenocytes obtained from the immunized F<sub>1</sub> mice were stimulated in vitro by splenocytes of C57BL/6 mice again to induce T cells having cytotoxicity specific to Qa-2 antigen. These T cells stimulated strongly not only normal lymphocytes of Qa-2<sup>+</sup> allogeneic mice but also tumor cells from C3H/He mice.

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Further, when splenocytes obtained from the F<sub>1</sub> mice immunized by lymphocytes of B6.K2 mice were stimulated in vitro by lymphocytes of B6.K2 mice, T cells having anti-Qa-2 cytotoxicity were induced. Cytotoxic activities were measured against target tumor cells, and shown in the following Table 10.

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Table 11:

Group	number of lymphocytes used for immunizing	number of lung metastases			
		C3H/He x B6.K1 F <sub>1</sub> mice		C3H/He x B6.K2 F <sub>1</sub> mice	
		mouse	number of nidi	mouse	number of nidi
1	$5 \times 10^6$	No.1	0	No.1	20
		No.2	0	No.2	6
		No.3	0	No.3	11
		No.4	0		
2	$5 \times 10^5$	No.1	0	No.1	9
		No.2	1	No.2	8
		No.3	0	No.3	10
		No.4	1		
3	$5 \times 10^4$	No.1	5	No.1	6
		No.2	8	No.2	12
		No.3	6	No.3	14
4	Control group with no immunization	No.1	5	No.1	9
		No.2	9	No.2	14
		No.3	9	No.3	10

Thus, it is evident that resistance against tumor cells can be induced by immunizing Qa-2<sup>-</sup> with Qa-2<sup>+</sup> allogeneic normal lymphocytes. Namely, immunoreaction against the Q7 gene product in Qa-2<sup>-</sup> mice has cross-reactivity with Q5 gene product, and it is evident that this cross-reactivity forms the above resistance against tumor cells.

#### Example 4:

As to non-classical histocompatibility class 1 antigens of human being, in some of the antigens, all nucleotide sequence of the gene has been decided already. It is known also that some of gene are inactive in normal adults.

Then, a polynucleotide, which has a nucleotide sequence specific to such a gene, is prepared and used as a primer in trying to amplify cDNAs prepared from human tumor cell strains (HL60, U937, Hmy2C1R and SKW-3) by PCR method. As shown in Fig. 11, nucleotide amplifications of expected length (shown by arrow) were detected.

This result shows that non-classical histocompatibility class 1 antigens, which are not activated in normal adult, were activated in human tumor cells.

As described above, resistance against tumor cells is induced in Qa-2<sup>-</sup> mice by immunizing the Qa-2<sup>-</sup> mice with Qa-2<sup>+</sup> allogeneic normal lymphocytes. Further, in tumor cells of Qa-2<sup>+</sup> mice origin, Q5 gene, which is not activated in normal adult mice, is activated, and therefore, immunoreaction specific to Q5 gene product can be induced by immune treatment using Q5 gene product, thus making it possible to induce resistance against autogenetic tumor cells.

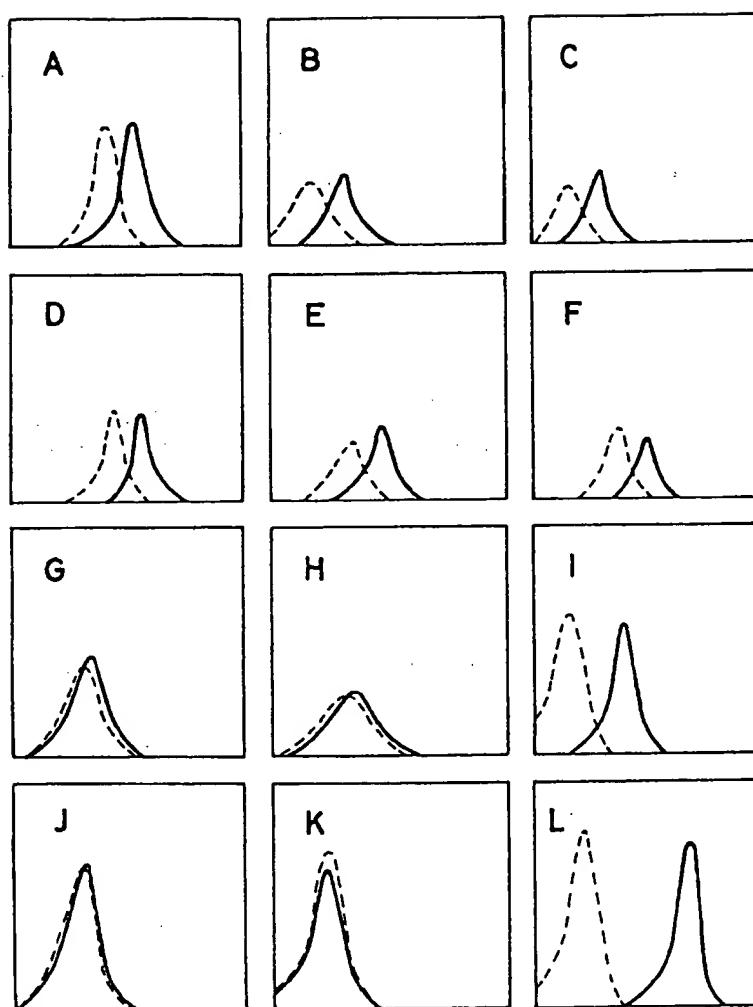
Moreover, in human beings also, non-classical histocompatibility class 1 antigens, which are not activated in normal adults, are activated in tumor cells.

Accordingly, an anti-tumor method and an anti-tumor agent are provide by the present invention. Namely, it is possible to induce resistance against tumor cells by administering a non-classical histocompatibility class 1 antigen, an antibody which identifies that antigen, or a cytotoxic lymphocyte.

#### Claims

1. An anti-tumor method comprising administering a non-classical histocompatibility class 1 antigen.
2. An anti-tumor method comprising administ ring an antibody which identifies a non-classical histocom-

Fig.1



( — ) : Qa-2-specific mAb 141-15.8  
 ( - - - ) : mAb specific to a bacterial component (negative control)

Fig.3

B

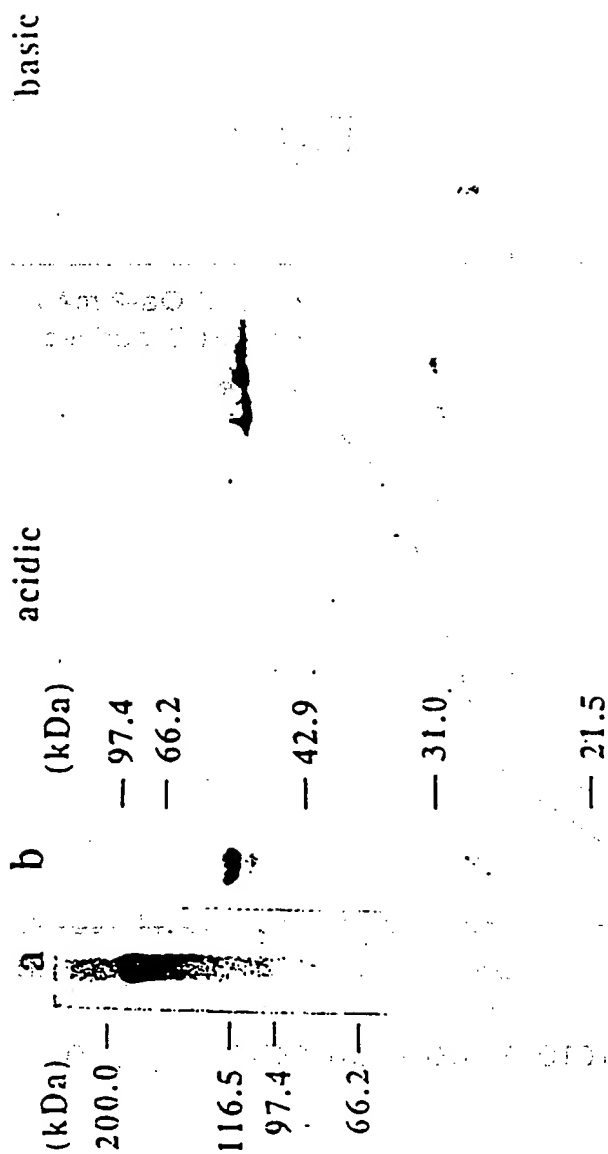


Fig.5

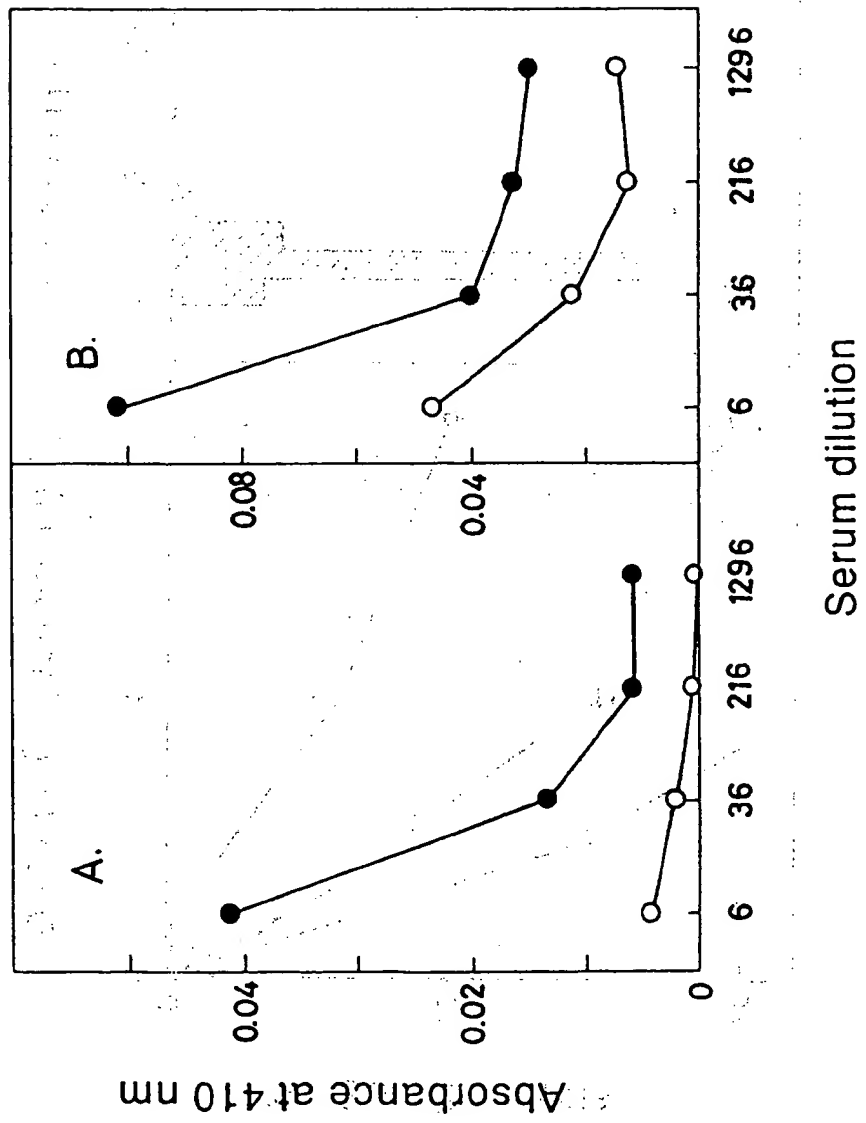


Fig.7

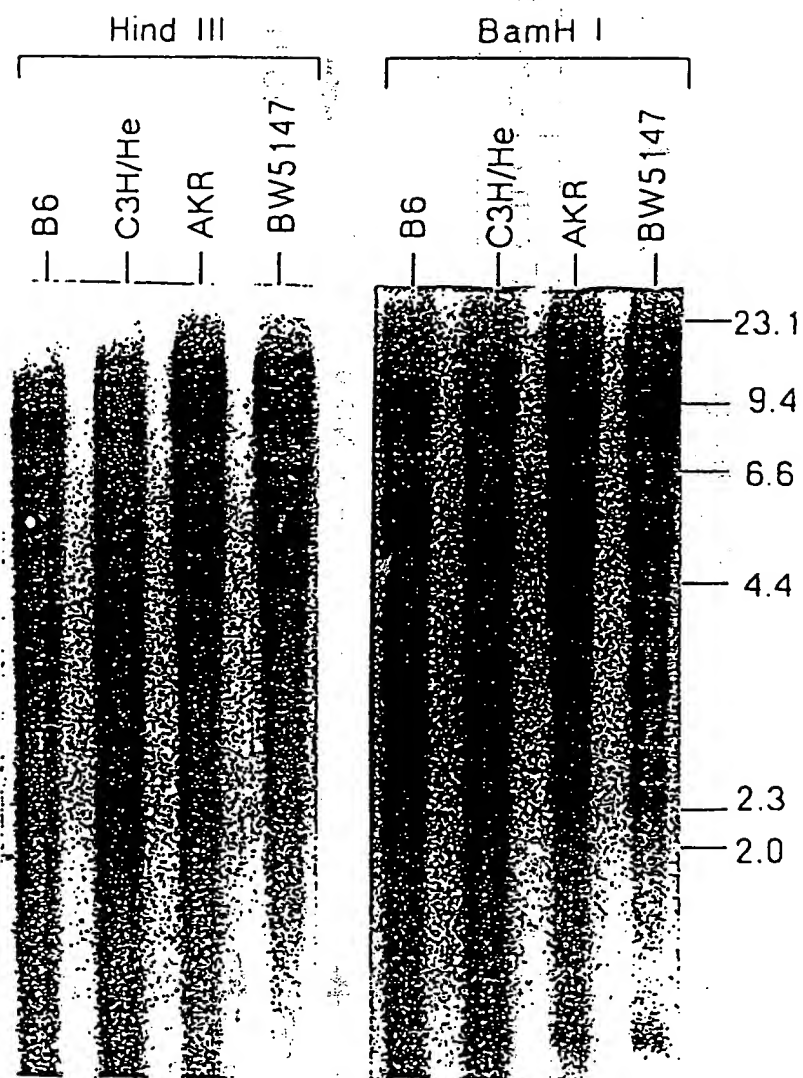


Fig.9

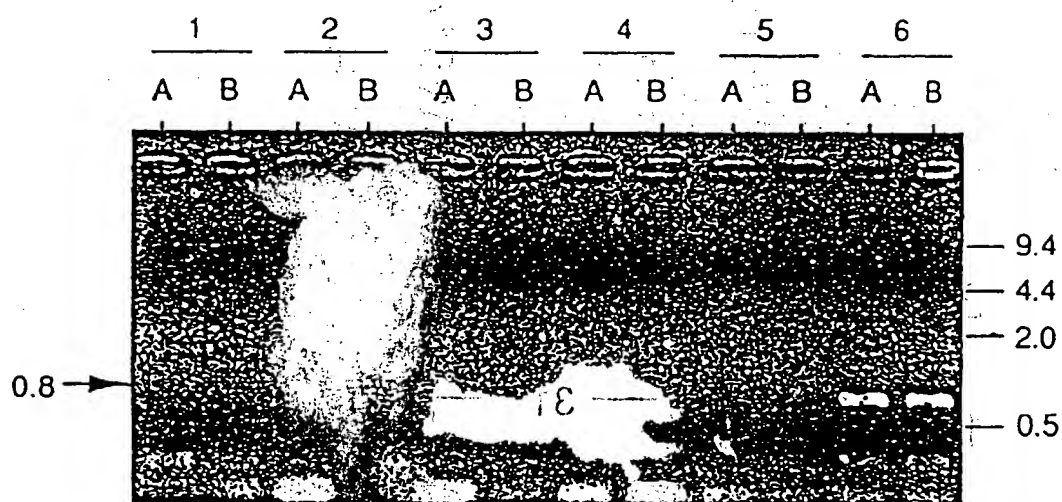
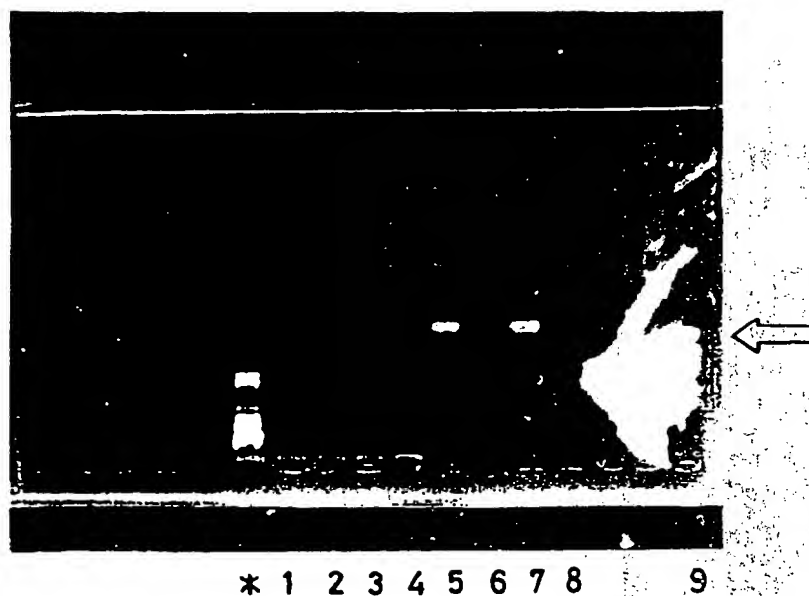




Fig.11



- \*: DNA-size marker  
1, 2: HL60  
3, 4: U937  
5, 6: Hmy2C1R  
7, 8: SKW-3  
9: Positive control (mouse tumor cell BW5147)

(19)



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(71) Applicant: **BIO DEFENCE INSTITUTE CO., LTD.**  
**Syouei-kannai Building, 22,**  
**Sumiyoshi-cho 2-chome,**  
**Naka-ku**  
**Yokohama-shi, Kanagawa(JP)**

(72) Inventor: **Egawa, Kohji**  
**1-11, Kamiyouga 3-chome,**  
**Setagaya-ku**  
**Tokyo(JP)**  
Inventor: **Sato, Ichiei**  
**2413, Ishihara-cho**  
**Takasaki-shi, Gunma(JP)**

(74) Representative: **Hering, Hartmut, Dipl.-Ing.**  
**Patentanwälte**  
**Berendt, Leyh & Hering**  
**Innere-Wiener-Strasse 20**  
**D-81667 München (DE)**

(54) **Anti-tumor method and anti-tumor agent.**

(57) An anti-tumor method and an anti-tumor agent using immunoreaction specific to tumor antigens expressed on the surface of tumor cells. A human non-classical histocompatibility class 1 antigen, an antibody which identify this antigen, or a cytotoxic lymphocyte is administered to a cancer patient as an immunotherapy to induce resistance against tumors.

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